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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| (21) International Application Number: PCT/DK99/00080 (22) International Filing Date: 25 February 1999 (25.02.99) (30) Priority Data: 0271/98 27 February 1998 (27.02.98) DK (71) Applicant: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors: KNUDSEN, Liselotte, Bjerre; Valby Langgade 49A, 1. tv., DK-2500 Valby (DK). HUUSFELDT, Per, Olaf; Applebys Plads 27,5. mf., DK-1411 Copenhagen K (DK). NIELSEN, Per, Franklin; Dalsø Park 59, DK-3500 Værløse (DK). KAARSHOLM, Niels, C.; Clausholmvej 38, DK-2720 Vanløse (DK). OLSEN, Helle, Birk; Skoleladden 23, DK-3450 Allerød (DK). THIM, Lars; Skiftevej 22, DK-2820 Gentofte (DK). BJØRN, Søren, Erik; Marie Grubbes Allé 47, DK-2800 Lyngby (DK). | | (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| (54) Title: GLP-2 DERIVATIVES WITH HELIX-CONTENT EXCEEDING 25 %, FORMING PARTIALLY STRUCTURED MICELLAR-LIKE AGGREGATES | | |
| (57) Abstract The present invention relates to a pharmaceutical composition comprising a GLP-2 derivative of improved solubility and/or stability, and to a method for improving the solubility and/or stability of GLP-2 or a fragment or an analogue thereof. | | |

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GLP-2 DERIVATIVES WITH HELIX-CONTENT EXCEEDING 25 %, FORMING PARTIALLY STRUCTURED MICEL-LAR-LIKE AGGREGATES

Field of the invention

- 5 The present invention relates to a pharmaceutical composition comprising a GLP-2 derivative of improved solubility and/or stability, and to a method for improving the solubility and/or stability of GLP-2 or a fragment or an analogue thereof.

Background of the invention

10

Peptides are widely used in medical practice, and since they can be produced by recombinant DNA technology it can be expected that their importance will increase also in the years to come. When native peptides or analogues thereof are used in therapy it is generally found that they have a high clearance. A high clearance of a therapeutic agent is inconvenient in cases
15 where it is desired to maintain a high blood level thereof over a prolonged period of time since repeated administrations will then be necessary. Examples of peptides which have a high clearance are: ACTH, corticotropin-releasing factor, angiotensin, calcitonin, insulin, glucagon, glucagon-like peptide-1, glucagon-like peptide-2, insulin-like growth factor-1, insulin-like growth factor-2, gastric inhibitory peptide, growth hormone-releasing factor, pituitary adenylate cyclase
20 activating peptide, secretin, enterogastrin, somatostatin, somatotropin, somatomedin, parathyroid hormone, thrombopoietin, erythropoietin, hypothalamic releasing factors, prolactin, thyroid stimulating hormones, endorphins, enkephalins, vasopressin, oxytocin, opioids and analogues thereof, superoxide dismutase, interferon, asparaginase, arginase, arginine deaminase, adenosine deaminase and ribonuclease. In some cases it is possible to influence the release profile
25 of peptides by applying suitable pharmaceutical compositions, but this approach has various shortcomings and is not generally applicable.

The amino acid sequence of GLP-2 and other preproglucagon fragments is given *i.a.* by Schmidt *et al.* (*Diabetologia* 28 704-707 (1985)). Little is known about the physical chemical
30 properties of GLP-2 but GLP-2 is expected, like GLP-1, to be a highly flexible and unstable molecule. GLP-2 and fragments thereof and analogues of GLP-2 and fragments thereof are potentially useful *i.a.* in regulation of appetite and in the treatment of small bowel syndrome. However, the high clearance limits the usefulness of these compounds, and thus there still is a need for improvements in this field.

We recently found that derivatisation of this relatively small and very flexible molecule resulted in compounds whose plasma profile were highly protracted and still had retained activity (PCT application No. DK97/00360).

Hitherto little was known about the physico-chemical and solution structural properties of GLP-2 derivatives. Such knowledge is a prerequisite for rational handling during e.g. production, purification and formulation work and is eventually important for understanding of the structural basis for the protraction mechanism.

Solubility limitations and the low stability against the actions of endogenous diaminopeptidyl peptidase limits the usefulness of GLP-2 derivatives, and thus there still is a need for improvements in this field. Accordingly, it is one object of the present invention to provide pharmaceutical solutions comprising GLP-2 derivatives with improved solubility and stability.

Summary of the invention

Preproglucagon, from which GLP-2 originates, is synthesized *i.a.* in the L-cells in the distal ileum, in the pancreas and in the brain. Processing of preproglucagon to give GLP-1 and GLP-2 occurs mainly in the L-cells. GLP-2 is a 33 amino acid residue peptide possibly in some tissue expended to 34 amino acid residues. A simple system is used to describe fragments, analogues and derivatives of GLP-2. Thus, for example, Lys²⁰GLP-2(1-33) designates a fragment of GLP-2 formally derived from GLP-2 by deleting the amino acid residues No. 34 and substituting the naturally occurring amino acid residue in position 20 (Arg) by Lys. Similarly, Arg³⁰Lys³⁵(N^t-tetradecanoyl)GLP-2(1-35) designates a derivative of a GLP-2 analogue formally derived from GLP-2 by C-terminal addition of a Lys residue, exchange of the naturally occurring amino acid residue in position 30 (Lys) with an Arg residue and tetradecanoylation of the ε-amino group of the Lys residue in position 35.

PCT application No. DK97/00360 describes various GLP-2 derivatives that are found to be very protracted. Whereas GLP-2 and GLP-2 analogues are molecules to which no defined solution structure can be ascribed, we found that some of these protracted GLP-2 derivatives may exist in a partially structured micellar-like aggregated form which is stable over a wide concentration range.

Circular Dichroism (CD) can be used to show that the GLP-2 derivatives have a certain partially structured conformation independent of their concentration. In contrast, for normal GLP-2 an increase in the helix content is seen with increasing concentration, from 10-15% to 30-35% (at 500 μ M concentration) in parallel with peptide self-association. For the GLP-2 derivatives
5 forming partially structured micellar-like aggregates in aqueous solution the helix content remains constant above 30% at concentrations of 10 μ M. The aggregated structured conformation is an inherent property of the derivative present in water or dilute aqueous buffer without the need for any additional structure-inducing components. Note that the CD signal is proportional to the average content of α -helix in the peptides, i.e., a CD value of -1 corresponds to 10% α -
10 helix content under these conditions.

Thus, in its broadest aspect, the present invention relates to a pharmaceutical composition comprising a GLP-2 derivative which has a helix content as measured by CD at 222 nm in H₂O at 22 ± 2 °C exceeding 25%, preferably in the range of 25% to 50%, at a peptide concentration
15 of about 10 μ M.

The size of the partially helical, micelle-like aggregates may be estimated by size-exclusion chromatography. Similarly, the apparent (critical micelle concentrations) CMC's of the peptides may be estimated from the concentration dependent fluorescence in the presence of
20 appropriate dyes (e.g. Brito, R. & Vaz, W. (1986) Anal. Biochem. **152**, 250-255).

That the derivatives have a partially structured micellar-like aggregate conformation in aqueous solutions makes them more soluble and stable in solution as compared to the native peptide. The increased solubility and stability can be seen by comparing the solubility after 9 days of
25 standing for a derivative and normal GLP-2(1-34) in a pharmaceutical formulation, e.g. 5 mM phosphate buffer, pH 6.9 added 0.1 M NaCl.

In the present text, the designation "an analogue" is used to designate a peptide wherein one or more amino acid residues of the parent peptide have been substituted by another amino
30 acid residue and/or wherein one or more amino acid residues of the parent peptide have been deleted and/or wherein one or more amino acid residues have been added to the parent peptide. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent peptide or both.

The term "derivative" is used in the present text to designate a peptide in which one or more of the amino acid residues of the parent peptide have been chemically modified, e.g. by alkylation, acylation, ester formation or amide formation.

- 5 The term "a GLP-2 derivative" is used in the present text to designate a derivative of GLP-2 or an analogue thereof. In the present text, the parent peptide from which such a derivative is formally derived is in some places referred to as the "GLP-2 moiety" of the derivative.

- 10 In a preferred embodiment, the present invention relates to pharmaceutical composition according to claim 1, wherein the concentration of GLP-2 derivative is not less than 0.5 mg/ml, preferably not less than about 5 mg/ml, more preferred not less than about 10 mg/ml and, preferably, not more than about 100 mg/ml.

- 15 The pharmaceutical composition of the invention preferably comprises a GLP-2 derivative wherein at least one amino acid residue of the parent peptide has a lipophilic substituent attached. More preferred are compositions comprising a GLP-2 derivative having a lipophilic substituent which is attached to any one of the amino acid residues in 20-34, preferably 30-34, most preferred 30.

- 20 The pharmaceutical composition according to the invention, preferably further comprises one or more of the following substances:

- a pharmaceutically acceptable vehicle or carrier;
- an isotonic agent, preferably selected from the group consisting of sodium chloride, mannitol and glycerol;
- 25 ◦ a preservative, preferably selected from the group consisting of phenol, m-cresol, methyl p-hydroxybenzoate, butyl p-hydroxybenzoate and benzyl alcohol;
- a buffer, preferably selected from the group consisting of sodium acetate, citrate, glycylglycine, histidine, 2-phenylethanol and sodium phosphate; and
- a surfactant capable of improving the solubility and/or the stability of the GLP-2 derivative, preferable selected from poloxamer 188, tween 20 and tween 80.

In a preferred embodiment, the pharmaceutical composition of the invention comprises a GLP-2 derivative wherein the lipophilic substituent comprises from 4 to 40 carbon atoms, preferably from 8 to 25 carbon atoms.

5 The lipophilic substituent is preferably attached to an amino acid residue in such a way that a carboxyl group of the lipophilic substituent forms an amide bond with an amino group of the amino acid residue, or, the lipophilic substituent is attached to an amino acid residue in such a way that an amino group of the lipophilic substituent forms an amide bond with a carboxyl group of the amino acid residue.

10 In a preferred embodiment the pharmaceutical composition according to the invention comprises a GLP-2 derivative wherein the lipophilic substituent is attached to the parent peptide by means of a spacer.

The spacer is preferably, in one embodiment, an unbranched alkane α,ω -dicarboxylic acid group having from 1 to 7 methylene groups, preferably two methylene groups, which form a bridge between an amino group of the parent peptide and an amino group of the lipophilic substituent.

The spacer is preferably, in another embodiment, an amino acid residue except Cys, or a dipeptide such as Gly-Lys or any unbranched alkane α,ω -aminoacid having from 1 to 7 methylene groups, preferably 2-4 methylene groups, which form a bridge between an amino group of the parent peptide and an amino group of the lipophilic substituent.

20 In a preferred embodiment, the lipophilic substituent comprises a partially or completely hydrogenated cyclopentanophenathrene skeleton.

In another preferred embodiment, the lipophilic substituent is a straight-chain or branched alkyl group.

25 The lipophilic substituent is preferably the acyl group of a straight-chain or branched fatty acid, the acyl group more preferably being:

- selected from the group comprising $\text{CH}_3(\text{CH}_2)_n\text{CO}-$, wherein n is 4 to 38, preferably $\text{CH}_3(\text{CH}_2)_6\text{CO}-$, $\text{CH}_3(\text{CH}_2)_8\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{10}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{12}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{14}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{16}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{18}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{20}\text{CO}-$ and $\text{CH}_3(\text{CH}_2)_{22}\text{CO}-$; or
- an acyl group of a straight-chain or branched alkane α,ω -dicarboxylic acid; or

- selected from the group comprising $\text{HOOC}(\text{CH}_2)_m\text{CO-}$, wherein m is from 4 to 38, preferably from 4 to 24, more preferred selected from the group comprising $\text{HOOC}(\text{CH}_2)_{14}\text{CO-}$, $\text{HOOC}(\text{CH}_2)_{16}\text{CO-}$, $\text{HOOC}(\text{CH}_2)_{18}\text{CO-}$, $\text{HOOC}(\text{CH}_2)_{20}\text{CO-}$ and $\text{HOOC}(\text{CH}_2)_{22}\text{CO-}$.

In another preferred embodiment, the lipophilic substituent is a group of the formula
 5 $\text{CH}_3(\text{CH}_2)_p((\text{CH}_2)_q\text{COOH})\text{CHNH-CO}(\text{CH}_2)_2\text{CO-}$, wherein p and q are integers and $p+q$ is an integer of from 8 to 33, preferably from 12 to 28.

In another preferred embodiment, the lipophilic substituent is a group of the formula
 $\text{CH}_3(\text{CH}_2)_r\text{CO-NHCH}(\text{COOH})(\text{CH}_2)_2\text{CO-}$, wherein r is an integer of from 10 to 24.

In another preferred embodiment, the lipophilic substituent is a group of the formula
 10 $\text{CH}_3(\text{CH}_2)_s\text{CO-NHCH}((\text{CH}_2)_2\text{COOH})\text{CO-}$, wherein s is an integer of from 8 to 24.

In another preferred embodiment, the lipophilic substituent is a group of the formula
 $\text{-NHCH}(\text{COOH})(\text{CH}_2)_4\text{NH-CO}(\text{CH}_2)_u\text{CH}_3$, wherein u is an integer of from 8 to 18.

In another preferred embodiment, the lipophilic substituent is a group of the formula
 $\text{-NHCH}(\text{COOH})(\text{CH}_2)_4\text{NH-COCH}((\text{CH}_2)_2\text{COOH})\text{NH-CO}(\text{CH}_2)_w\text{CH}_3$, wherein w is an integer of
 15 from 10 to 16.

In another preferred embodiment, the lipophilic substituent is a group of the formula
 $\text{-NHCH}(\text{COOH})(\text{CH}_2)_4\text{NH-CO}(\text{CH}_2)_2\text{CH}(\text{COOH})\text{NH-CO}(\text{CH}_2)_x\text{CH}_3$, wherein x is an integer of
 from 10 to 16.

In another preferred embodiment, the lipophilic substituent is a group of the formula
 20 $\text{-NHCH}(\text{COOH})(\text{CH}_2)_4\text{NH-CO}(\text{CH}_2)_2\text{CH}(\text{COOH})\text{NH-CO}(\text{CH}_2)_y\text{CH}_3$, wherein y is zero or an integer of from 1 to 22.

In a preferred embodiment the pharmaceutical composition according to the invention, comprises a GLP-2 derivative wherein the parent peptide is selected from the group comprising GLP-2(1-30); GLP-2(1-31); GLP-2(1-32); GLP-2(1-33); GLP-2(1-34) and GLP-2(1-35).

25

In a further preferred embodiment the pharmaceutical composition according to the invention, comprises a GLP-2 derivative wherein the parent peptide has the following amino acid sequence:

$X^1 H X^2 D G S F S D E M N T X^3 L D X^4 L A X^5 X^6 D F I N W L X^7 X^8 T K I T D X^9$

wherein X^1 is NH_2 , DFPEEVAIVEELGRR, DFPEEV TIVEELGRR, DFPEEVNIVEELRRR, or a fragment thereof,

5 X^2 is Ala or Gly,

X^3 is Ile or Val,

X^4 is Asn, Ser or His,

X^5 is Ala or Thr,

X^6 is Arg or Lys,

10 X^7 is Ile or Leu,

X^8 is Gln or His, or

X^9 is OH, Lys, Arg, Arg-Lys, Lys-Arg, Arg-Arg or Lys-Lys.

15 The pharmaceutical composition according to the invention preferably comprises a GLP-2 derivative wherein a total of up to fifteen, preferably up to ten, more preferably up to six, amino acid residues have been exchanged with any α -amino acid residue which can be coded for by the genetic code.

The parent peptide is most preferably selected from the group comprising:

Lys²⁰GLP-2(1-33);

20 Lys²⁰Arg³⁰GLP-2(1-33);

Arg³⁰Lys³⁴GLP-2(1-34);

Arg³⁰Lys³⁵GLP-2(1-35);

Arg^{30,35}Lys²⁰GLP-2(1-35);

Arg³⁵GLP-2(1-35).

25

In a further preferred embodiment, the pharmaceutical composition of the present invention comprises a GLP-2 derivative which is selected from the group comprising

Lys²⁰(N^ε-tetradecanoyl)GLP-2(1-33);

30 Lys^{20,30}-bis(N^ε-tetradecanoyl)GLP-2(1-33);

Lys²⁰(N^ε-tetradecanoyl)Arg³⁰GLP-2(1-33);

Arg³⁰Lys³⁵(N^ε-tetradecanoyl)GLP-2(1-35);

Arg^{30,35}Lys²⁰(N^ε-tetradecanoyl)GLP-2(1-35);

Arg³⁵Lys³⁰(N^ε-tetradecanoyl)GLP-2(1-35);
 Arg³⁰Lys³⁴(N^ε-tetradecanoyl)GLP-2(1-34);
 Lys²⁰(N^ε-(ω-carboxynonadecanoyl))GLP-2(1-33);
 Lys^{20,30}-bis(N^ε-(ω-carboxynonadecanoyl))GLP-2(1-33);
 5 Lys²⁰(N^ε-(ω-carboxynonadecanoyl))Arg³⁰GLP-2(1-33);
 Arg³⁰Lys³⁵(N^ε-(ω-carboxynonadecanoyl))GLP-2(1-35);
 Arg^{30,35}Lys²⁰(N^ε-(ω-carboxynonadecanoyl))GLP-2(1-35);
 Arg³⁵Lys³⁰(N^ε-(ω-carboxynonadecanoyl))GLP-2(1-35); and
 Arg³⁰Lys³⁴(N^ε-(ω-carboxynonadecanoyl))GLP-2(1-34).

10

The present invention furthermore relates to a method for improving the solubility and/or stability of GLP-2 or a fragment or an analogue thereof, characterised in that a lipophilic substituent is introduced on any one of the amino acid residues of the parent peptide.

By this method the lipophilic substituent is preferably introduced on any one of the amino acid
 15 residues in position 20-34, preferably 30-34, most preferred 30.

The lipophilic substituent preferably comprises from 4 to 40 carbon atoms, more preferably from 8 to 25 carbon atoms.

In a preferred embodiment the lipophilic substituent is the acyl group of a straight-chain or branched fatty acid; preferably selected from the group comprising CH₃(CH₂)_nCO-, wherein n is
 20 4 to 38, preferably CH₃(CH₂)₆CO-, CH₃(CH₂)₈CO-, CH₃(CH₂)₁₀CO-, CH₃(CH₂)₁₂CO-,
 CH₃(CH₂)₁₄CO-, CH₃(CH₂)₁₆CO-, CH₃(CH₂)₁₈CO-, CH₃(CH₂)₂₀CO- and CH₃(CH₂)₂₂CO-.

The GLP-2 parent peptide is preferably GLP-2(1-30); GLP-2(1-31); GLP-2(1-32); GLP-2(1-33); GLP-2(1-34) and GLP-2(1-35).

25 Detailed description of the invention

To obtain a satisfactory protracted profile of action of the GLP-2 derivative, the lipophilic substituent attached to the GLP-2 moiety preferably comprises 4-40 carbon atoms, in particular 8-25 carbon atoms. The lipophilic substituent may be attached to an amino group of the GLP-2
 30 moiety by means of a carboxyl group of the lipophilic substituent which forms an amide bond with an amino group of the amino acid to which it is attached. As an alternative, the lipophilic

substituent may be attached to said amino acid in such a way that an amino group of the lipophilic substituent forms an amide bond with a carboxyl group of the amino acid. As a further option, the lipophilic substituent may be linked to the GLP-2 moiety via an ester bond. Formally, the ester can be formed either by reaction between a carboxyl group of the GLP-2 moiety and a hydroxyl group of the substituent-to-be or by reaction between a hydroxyl group of the GLP-2 moiety and a carboxyl group of the substituent-to-be. As a further alternative, the lipophilic substituent can be an alkyl group which is introduced into a primary amino group of the GLP-2 moiety.

In one preferred embodiment of the invention, the lipophilic substituent is attached to the GLP-2 moiety by means of a spacer in such a way that a carboxyl group of the spacer forms an amide bond with an amino group of the GLP-2 moiety. Examples of suitable spacers are succinic acid, Lys, Glu or Asp, or a dipeptide such as Gly-Lys. When the spacer is succinic acid, one carboxyl group thereof may form an amide bond with an amino group of the amino acid residue, and the other carboxyl group thereof may form an amide bond with an amino group of the lipophilic substituent. When the spacer is Lys, Glu or Asp, the carboxyl group thereof may form an amide bond with an amino group of the amino acid residue, and the amino group thereof may form an amide bond with a carboxyl group of the lipophilic substituent. When Lys is used as the spacer, a further spacer may in some instances be inserted between the ϵ -amino group of Lys and the lipophilic substituent. In one preferred embodiment, such a further spacer is succinic acid which forms an amide bond with the ϵ -amino group of Lys and with an amino group present in the lipophilic substituent. In another preferred embodiment such a further spacer is Glu or Asp which forms an amide bond with the ϵ -amino group of Lys and another amide bond with a carboxyl group present in the lipophilic substituent, that is, the lipophilic substituent is a N^ε-acylated lysine residue.

In another preferred embodiment of the present invention, the lipophilic substituent has a group which can be negatively charged. One preferred group which can be negatively charged is a carboxylic acid group.

The parent peptide can be produced by a method which comprises culturing a host cell containing a DNA sequence encoding the peptide and capable of expressing the peptide in a suitable nutrient medium under conditions permitting the expression of the peptide, after which the resulting peptide is recovered from the culture.

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The peptide produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gelfiltration chromatography, affinity chromatography, or the like, dependent on the type of peptide in question.

The DNA sequence encoding the parent peptide may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the peptide by hybridisation using synthetic oligonucleotide probes in accordance with standard techniques (see, for example, Sambrook, J, Fritsch, EF and Maniatis, T, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989). The DNA sequence encoding the peptide may also be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by Beaucage and Caruthers, *Tetrahedron Letters* 22 (1981), 1859 - 1869, or the method described by Matthes *et al.*, *EMBO Journal* 3 (1984), 801 - 805. The DNA sequence may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki *et al.*, *Science* 239 (1988), 487 - 491.

The DNA sequence may be inserted into any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the peptide is operably linked to additional segments required for transcription of the DNA, such as a pro-

moter. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the peptide of the invention in a variety of host cells are well known in the art, cf. for instance Sambrook *et al.*, *supra*.

The DNA sequence encoding the peptide may also, if necessary, be operably connected to a suitable terminator, polyadenylation signals, transcriptional enhancer sequences, and translational enhancer sequences. The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question.

The vector may also comprise a selectable marker, *e.g.* a gene the product of which complements a defect in the host cell or one which confers resistance to a drug, *e.g.* ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate.

To direct a parent peptide of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the peptide in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be that normally associated with the peptide or may be from a gene encoding another secreted protein.

The procedures used to ligate the DNA sequences coding for the present peptide, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook *et al.*, *supra*).

The host cell into which the DNA sequence or the recombinant vector is introduced may be any cell which is capable of producing the present peptide and includes bacteria, yeast, fungi and higher eukaryotic cells. Examples of suitable host cells well known and used in the art are, without limitation, *E. coli*, *Saccharomyces cerevisiae*, or mammalian BHK or CHO cell lines.

The GLP-2 derivatives of the invention can be prepared by introducing the lipophilic substituent into the parent GLP-2 or GLP-2 analogue using methods known *per se*, see for example WO 95/07931, the contents of which is hereby incorporated in its entirety by reference.

- 5 N^ε-acylation of a Lys residue can be carried out by using an activated amide of the acyl group to be introduced as the acylating agent, e.g. the amide with benzotriazole. The acylation is carried out in a polar solvent in the presence of a base.

10 Preparation and administration of the compositions

- Pharmaceutical compositions containing a GLP-2 derivative according to the present invention may be administered parenterally or orally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by
15 means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the GLP-2 derivative in the form of a nasal or pulmonary spray. As a still further option, the pharmaceutical compositions containing a the GLP-2 derivatives of the invention can also be adapted to transdermal administration, e.g. from a
20 patch, optionally a iontophoretic patch, or transmucosal, e.g. bucal, administration.

Pharmaceutical compositions containing a GLP-2 derivative of the present invention may be prepared by conventional techniques, e.g. as described in Remington's *Pharmaceutical Sciences*, 1985 or in Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

- 25 Thus, the injectable compositions of the GLP-2 derivative of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

- 30 According to one procedure, the GLP-2 derivative is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted - if necessary - using an acid, e.g. hydrochloric acid, or a base, e.g. aqueous sodium hydroxide as

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needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

A composition for nasal administration of certain peptides may, for example, be prepared as
5 described in European Patent No. 272097 (to Novo Nordisk A/S) or in WO 93/18785.

According to one preferred embodiment of the present invention, the pharmaceutical compositions containing a GLP-2 derivative is provided in the form of a composition suitable for administration by injection. Such a composition can either be an injectable solution ready for use or it
10 can be an amount of a solid composition, e.g. a lyophilised product, which has to be dissolved in a solvent before it can be injected. The injectable solution preferably contains not less than about 0.5 mg/ml, preferably not less than about 5 mg/ml, more preferred not less than about 10 mg/ml of the GLP-2 derivative and, preferably, not more than about 100 mg/ml of the GLP-2 derivative.

15 The pharmaceutical compositions containing a GLP-2 derivative of this invention can be used in the treatment of various diseases, including obesity, small bowel syndrome, Crohn's disease, ileitis, intestinal inflammation, gastric and duodenal ulceration, inflammatory bowel disease (IBD) and intestinal cancer damage therapy. The particular GLP-2 derivative to be used and
20 the optimal dose level for any patient will depend on the disease to be treated and on a variety of factors including the efficacy of the specific peptide derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case. It is recommended that the dosage of the GLP-2 derivative of this invention be determined for each individual patient by those skilled in the art.

25

Example 1

Synthesis of Lys³⁰ (N^ε-(γ-glutamyl(N^α-tetradecanoyl))) hGLP-2.

To a mixture of hGLP-2-OH (5 mg, 1.33 μmol), EDPA (4.8 mg, 37.2 μmol), NMP (0.7 ml) and water (0.35 ml) was added a solution of Myr-Glu(ONSu)-OBu^t (2 mg, 4 μmol), prepared
30 as described in PCT application no. PCT/DK97/00340, in NMP (51 μl). The reaction mixture was gently shaken for 5 min., and then allowed to stand for an additional 110 min. at room temperature. The reaction was quenched by the addition of a solution of glycine (2.2 mg, 29.3 μmol) in water (22 μl). A 0.5% aqueous solution of ammonium acetate (15 ml) was added, and the resulting mixture eluted onto a Varian 5g C8 Mega Bond Elut®, the immobilised
35 compound washed with 5% aqueous acetonitril (20 ml), and finally liberated from the car-

tridge by elution with TFA (20 ml). The eluate was concentrated *in vacuo*, and the residue purified by column chromatography using a cyanopropyl column (Zorbax 300SB-CN) and a standard acetonitril/TFA system. The column was heated to 65°C and the acetonitril gradient was 0-100% in 60 minutes. The title compound (0.1 mg, 1.8 %) was isolated, and the product was analysed by PDMS. The m/z value for the protonated molecular ion was found to be 4107.8 ± 3 . The resulting molecular weight is thus 4106.8 ± 3 amu (theoretical value 4106 amu).

CLAIMS

1. A pharmaceutical composition comprising a GLP-2 derivative which has a helix content as
5 measured by CD at 222 nm in H₂O at 22 ± 2 °C exceeding 25%, preferably in the range of 25% to 50%, at a peptide concentration of about 10 µM .
2. A pharmaceutical composition according to claim 1, wherein the concentration of GLP-2 derivative is not less than 0.5 mg/ml, preferably not less than about 5 mg/ml, more preferred not less than about 10 mg/ml and, preferably, not more than about 100 mg/ml.
- 10 3. A pharmaceutical composition according to claim 1 or 2, comprising a GLP-2 derivative wherein at least one amino acid residue of the parent peptide has a lipophilic substituent attached.
4. A pharmaceutical composition according to claim 3, comprising a GLP-2 derivative having a lipophilic substituent which is attached to any one of the amino acid residues in position
15 20-34, preferably 30-34, most preferred 30.
5. A pharmaceutical composition according to any one of the preceding claims, further comprising a pharmaceutically acceptable vehicle or carrier.
6. A pharmaceutical composition according to any one of the preceding claims, further comprising an isotonic agent, preferably selected from the group consisting of sodium chloride,
20 mannitol and glycerol.
7. A pharmaceutical composition according to any one of the preceding claims, further comprising a preservative, preferably selected from the group consisting of phenol, m-cresol, methyl p-hydroxybenzoate, butyl p-hydroxybenzoate and benzyl alcohol.
8. A pharmaceutical composition according to any one of the preceding claims, further comprising a buffer, preferably selected from the group consisting of sodium acetate, citrate,
25 glycylglycine, histidine, 2-phenylethanol and sodium phosphate.
9. A pharmaceutical composition according to any one of the preceding claims, further comprising a surfactant capable of improving the solubility and/or the stability of the GLP-2 derivative, preferable selected from poloxamer 188, tween 20 and tween 80.

10. A pharmaceutical composition according to any one of the preceding claims, comprising a GLP-2 derivative wherein the lipophilic substituent comprises from 4 to 40 carbon atoms, preferably from 8 to 25 carbon atoms.
- 5 11. A pharmaceutical composition according to any one of the preceding claims, comprising a GLP-2 derivative wherein a lipophilic substituent is attached to an amino acid residue in such a way that a carboxyl group of the lipophilic substituent forms an amide bond with an amino group of the amino acid residue.
- 10 12. A pharmaceutical composition according to any one of the claims 1-11, comprising a GLP-2 derivative wherein a lipophilic substituent is attached to an amino acid residue in such a way that an amino group of the lipophilic substituent forms an amide bond with a carboxyl group of the amino acid residue.
13. A pharmaceutical composition according to any one of the preceding claims, comprising a GLP-2 derivative wherein the lipophilic substituent is attached to the parent peptide by means of a spacer.
- 15 14. A pharmaceutical composition according to claim 13, wherein the spacer is an unbranched alkane α,ω -dicarboxylic acid group having from 1 to 7 methylene groups, preferably two methylene groups, which form a bridge between an amino group of the parent peptide and an amino group of the lipophilic substituent.
- 20 15. A pharmaceutical composition according to claim 13, wherein the spacer is an amino acid residue except Cys, or a dipeptide such as Gly-Lys or any unbranched alkane α,ω -aminoacid having from 1 to 7 methylene groups, preferably 2-4 methylene groups, which form a bridge between an amino group of the parent peptide and an amino group of the lipophilic substituent.
- 25 16. A pharmaceutical composition according to any one of the preceding claims, comprising a GLP-2 derivative wherein the lipophilic substituent comprises a partially or completely hydrogenated cyclopentanophenathrene skeleton.
17. A pharmaceutical composition according to any one of claims 3 to 15, wherein the lipophilic substituent is a straight-chain or branched alkyl group.

18. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-2 derivative wherein the lipophilic substituent is the acyl group of a straight-chain or branched fatty acid.
- 5 19. A pharmaceutical composition according to claim 18 wherein the acyl group is selected from the group comprising $\text{CH}_3(\text{CH}_2)_n\text{CO}-$, wherein n is 4 to 38, preferably $\text{CH}_3(\text{CH}_2)_6\text{CO}-$, $\text{CH}_3(\text{CH}_2)_8\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{10}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{12}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{14}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{16}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{18}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{20}\text{CO}-$ and $\text{CH}_3(\text{CH}_2)_{22}\text{CO}-$.
- 10 20. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-2 derivative wherein the lipophilic substituent is an acyl group of a straight-chain or branched alkane α,ω -dicarboxylic acid.
21. A pharmaceutical composition according to claim 20 wherein the acyl group is selected from the group comprising $\text{HOOC}(\text{CH}_2)_m\text{CO}-$, wherein m is from 4 to 38, preferably from 4 to 24, more preferred selected from the group comprising $\text{HOOC}(\text{CH}_2)_{14}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{16}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{18}\text{CO}-$, $\text{HOOC}(\text{CH}_2)_{20}\text{CO}-$ and $\text{HOOC}(\text{CH}_2)_{22}\text{CO}-$.
- 15 22. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-2 derivative wherein the lipophilic substituent is a group of the formula $\text{CH}_3(\text{CH}_2)_p((\text{CH}_2)_q\text{COOH})\text{CHNH-CO}(\text{CH}_2)_2\text{CO}-$, wherein p and q are integers and p+q is an integer of from 8 to 33, preferably from 12 to 28.
- 20 23. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-2 derivative, wherein the lipophilic substituent is a group of the formula $\text{CH}_3(\text{CH}_2)_r\text{CO-NHCH}(\text{COOH})(\text{CH}_2)_2\text{CO}-$, wherein r is an integer of from 10 to 24.
24. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-2 derivative, wherein the lipophilic substituent is a group of the formula $\text{CH}_3(\text{CH}_2)_s\text{CO-NHCH}((\text{CH}_2)_2\text{COOH})\text{CO}-$, wherein s is an integer of from 8 to 24.
- 25 25. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-2 derivative, wherein the lipophilic substituent is a group of the formula $-\text{NHCH}(\text{COOH})(\text{CH}_2)_4\text{NH-CO}(\text{CH}_2)_u\text{CH}_3$, wherein u is an integer of from 8 to 18.
26. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-2 derivative, wherein the lipophilic substituent is a group of the formula

-NHCH(COOH)(CH₂)₄NH-COCH((CH₂)₂COOH)NH-CO(CH₂)_wCH₃, wherein w is an integer of from 10 to 16.

27. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-2 derivative, wherein the lipophilic substituent is a group of the formula

-NHCH(COOH)(CH₂)₄NH-CO(CH₂)₂CH(COOH)NH-CO(CH₂)_xCH₃, wherein x is an integer of from 10 to 16.

28. A pharmaceutical composition according to any one of claims 3 to 15, comprising a GLP-2 derivative wherein the lipophilic substituent is a group of the formula

-NHCH(COOH)(CH₂)₄NH-CO(CH₂)₂CH(COOH)NH-CO(CH₂)_yCH₃, wherein y is zero or an integer of from 1 to 22.

29. A pharmaceutical composition according to any one of the preceding claims, comprising a GLP-2 derivative wherein the parent peptide is selected from the group comprising GLP-2(1-30); GLP-2(1-31); GLP-2(1-32); GLP-2(1-33); GLP-2(1-34) and GLP-2(1-35).

30. A pharmaceutical composition according to any one of the preceding claims, comprising a GLP-2 derivative wherein the parent peptide has the following amino acid sequence

X¹ H X² D G S F S D E M N T X³ L D X⁴ L A X⁵ X⁶ D F I N W L X⁷ X⁸ T K I T D X⁹ wherein X¹ is NH₂, DFPEEVAIVEELGRR, DFPEEVTIVEELGRR, DFPEEVNIVEELRRR, or a fragment thereof,

X² is Ala or Gly, X³ is Ile or Val, X⁴ is Asn, Ser or His, X⁵ is Ala or Thr, X⁶ is Arg or Lys, X⁷ is Ile or Leu, X⁸ is Gln or His, or X⁹ is OH, Lys, Arg, Arg-Lys, Lys-Arg, Arg-Arg or Lys-Lys

31. A pharmaceutical composition according to any of the preceding claims comprising a GLP-2 derivative wherein a total of up to fifteen, preferably up to ten, more preferably up to six, amino acid residues have been exchanged with any α-amino acid residue which can be coded for by the genetic code.

32. A pharmaceutical composition according to any of the preceding claims, comprising a GLP-2 derivative wherein the parent peptide is selected from the group comprising Lys²⁰GLP-2(1-33); Lys²⁰Arg³⁰GLP-2(1-33); Arg³⁰Lys³⁴GLP-2(1-34); Arg³⁰Lys³⁵GLP-2(1-35); Arg^{30,35}Lys²⁰GLP-2(1-35); Arg³⁵GLP-2(1-35).

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 99/00080

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 58/26, C07K 14/605

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, MEDLINE, BIOSIS, EMBASE, SCISEARCH

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| X | Pharmaceutical Research, Volume 15, No 2, 1998, Dean K. Clodfelter et al, "Effects of Non-Covalent Self-Association on the Subcutaneous Absorption of a Therapeutic Peptide", page 254 - page 262, abstract, figure 1, table 1 | 33-38 |
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☒ Further documents are listed in the continuation of Box C.

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33. A method for improving the solubility and/or stability of GLP-2 or a fragment or an analogue thereof, characterised in that a lipophilic substituent is introduced on any one of the amino acid residues of the parent peptide.
34. A method according to claim 33, wherein a lipophilic substituent is introduced on any one
5 of the amino acid residues in position 20-34, preferably 30-34, most preferred 30.
35. A method according to claim 33 or 34, wherein the lipophilic substituent comprises from 4 to 40 carbon atoms, preferably from 8 to 25 carbon atoms.
36. A method according to any one of claim 33 to 35, wherein the lipophilic substituent is the acyl group of a straight-chain or branched fatty acid.
- 10 37. A method according to any one of claim 36, wherein the acyl group is selected from the group comprising $\text{CH}_3(\text{CH}_2)_n\text{CO}-$, wherein n is 4 to 38, preferably $\text{CH}_3(\text{CH}_2)_6\text{CO}-$, $\text{CH}_3(\text{CH}_2)_8\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{10}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{12}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{14}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{16}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{18}\text{CO}-$, $\text{CH}_3(\text{CH}_2)_{20}\text{CO}-$ and $\text{CH}_3(\text{CH}_2)_{22}\text{CO}-$.
- 15 38. A method according to any one of claims 33 to 37, wherein the parent peptide is selected from the group comprising $\text{Lys}^{20}\text{GLP-2(1-33)}$; $\text{Lys}^{20}\text{Arg}^{30}\text{GLP-2(1-33)}$; $\text{Arg}^{30}\text{Lys}^{34}\text{GLP-2(1-34)}$; $\text{Arg}^{30}\text{Lys}^{35}\text{GLP-2(1-35)}$; $\text{Arg}^{30,35}\text{Lys}^{20}\text{GLP-2(1-35)}$; $\text{Arg}^{35}\text{GLP-2(1-35)}$.
39. Use of a pharmaceutical composition according to any one of claims 1 to 32 for the preparation of a medicament for treating obesity.
- 20 40. Use of a pharmaceutical composition according to any one of claims 1 to 32 for the preparation of a medicament for treating small bowel syndrome, Crohn's disease, ileitis, intestinal inflammation, gastric and duodenal ulceration, inflammatory bowel disease (IBD) and intestinal cancer damage therapy.

INTERNATIONAL SEARCH REPORT

Information on patent family members

03/05/99

International application No.

PCT/DK 99/00080

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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International application No.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| (51) International Patent Classification ⁶ : A61K 38/26, C07K 14/605 | A1 | (11) International Publication Number: WO 99/43361 (43) International Publication Date: 2 September 1999 (02.09.99) |
| (21) International Application Number: PCT/DK99/00080 (22) International Filing Date: 25 February 1999 (25.02.99) (30) Priority Data: 0271/98 27 February 1998 (27.02.98) DK (71) Applicant: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors: KNUDSEN, Liselotte, Bjerre; Valby Langgade 49A, 1. tv., DK-2500 Valby (DK). HUUSFELDT, Per, Olaf; Applebys Plads 27,5. mf., DK-1411 Copenhagen K (DK). NIELSEN, Per, Franklin; Dalsø Park 59, DK-3500 Værløse (DK). KAARSHOLM, Niels, C.; Clausholmvej 38, DK-2720 Vanløse (DK). OLSEN, Helle, Birk; Skolelodden 23, DK-3450 Allerød (DK). THIM, Lars; Skiftevej 22, DK-2820 Gentofte (DK). BJØRN, Søren, Erik; Marie Grubbes Allé 47, DK-2800 Lyngby (DK). | | (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With a revised version of the international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the revised version of the international search report: 14 October 1999 (14.10.99) |
| (54) Title: GLP-2 DERIVATIVES WITH HELIX-CONTENT EXCEEDING 25 %, FORMING PARTIALLY STRUCTURED MICEL-LAR-LIKE AGGREGATES | | |
| (57) Abstract The present invention relates to a pharmaceutical composition comprising a GLP-2 derivative of improved solubility and/or stability, and to a method for improving the solubility and/or stability of GLP-2 or a fragment or an analogue thereof. | | |

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| DK | Denmark | LK | Sri Lanka | SE | Sweden | | |
| EE | Estonia | LR | Liberia | SG | Singapore | | |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 99/00080

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 38/26, C07K 14/605

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, MEDLINE, BIOSIS, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

17 August 1999

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00080

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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02/08/99

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